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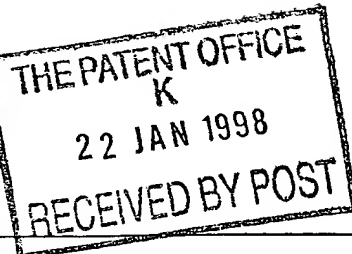
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06584809003

29/1/98

4. Title of the invention

7482391001

SEQUENCE OF PROTEIN-PROTEIN, PROTEIN-NON PROTEIN AND
PROTEIN-PROTEIN INTERACTIONS

5. Name of your agent (if you have one)

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Number of earlier application

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- a) any applicant named in part 3 is not an inventor, or
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Description

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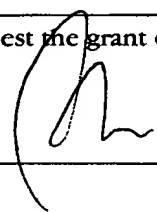
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SELECTION OF PROTEIN-PROTEIN, PROTEIN-NON PROTEIN AND PROTEIN-DEFINED PROTEIN INTERACTIONS

The present invention relates to new screening methods to isolate genes encoding binding polypeptides from DNA libraries. In particular, the method relates to screening methods for novel polypeptide-polypeptide interactions. The method uses interaction of a polypeptide with a target molecule or 2 (or more) polypeptides to produce a detectable or selectable property which allows isolation of genes encoding the binding polypeptides(s).

There is considerable interest in "protein display" methods for isolating polypeptides with useful phenotypes from gene libraries encoding a large mixture of polypeptides whereby the corresponding genes can be recovered swiftly and easily. For example, there has been considerable success in identifying useful peptides or antibody fragments from DNA libraries using bacteriophage display methods to produce polypeptides fused to phage polypeptides which can bind to ligands of interest. In addition, there has been success in identifying useful polypeptides displayed on the surface of bacteria. More recently, there has been an interest in evolving new polypeptides displayed on bacteriophage and bacteria by successive rounds of gene mutation and selection for polypeptides binding to the ligand of interest.

Whilst display of polypeptides on microorganisms has provided a link between a useful binding phenotype and genotype, these methods have largely been limited to detection of polypeptides by virtue of binding to a ligand which is or becomes immobilised thus allowing for separation of the microorganisms displaying a useful binding phenotype. The method has, in practice, been limited by non-specific binding to the solid phase. In addition, where the ligand is another polypeptide, it is desirable to simultaneously examine binding with different variants or libraries of this ligand without selecting the first binding polypeptide(s) and then making a DNA library encoding variants of ligands to screen against the first selected polypeptide. One technology to address these problems has been proposed by Krebber et al., J Mol Biol, vol 268 (1997) p619-630. This method uses a defective bacteriophage with the polypeptide displayed in place of two domain of the gIIIp polypeptide and the ligand (or other polypeptide) provided in association with these two domains. Upon binding of the polypeptide to it's ligand either externally or internally within the bacterial cell, the bacteriophage infectivity is restored thus providing a biological selection for interacting binding pairs. Disadvantages of the method of Krebber et al include the low infectivity of restored bacteriophage and the binding of free ligand-associated gIIIp to the bacterial F pilus in competition with infective phage. There is therefore a need for improved polypeptide display methods which do not require solid phase separation and which allow the simultaneous selection of binding pairs from tandem DNA libraries encoding polypeptides.

The present invention is based upon the binding of a polypeptide to a ligand or a polypeptide to another polypeptide or a polypeptide to a non-protein such as DNA, RNA or a chemical in such a way so as to generate a detectable or selectable property which then allows isolation of genes encoding the binding polypeptides(s).

One embodiment of the invention is based upon the binding of a polypeptide to a ligand (or other polypeptide) whereby molecular tags on the polypeptide and ligand can be used as the basis for molecular separation of the bound polypeptide-ligand from unbound polypeptide and ligand whereby the polypeptide is still associated with its gene (or gene transcript). For selection of a polypeptide which binds to a ligand, a particularly favourable method is to provide molecular tags on both the polypeptide and its ligand whereby methods are then applied for isolation of complexes containing both molecular tags. For example, using the technique of ribosome display, the mRNA may be tagged with a RNA binding protein (such as HIV tat protein) whilst the ligand (if a polypeptide) may be tagged with a polyhistidine tail such that consecutive passage of a library of mRNA-ribosome-polypeptide complexes over affinity matrices comprising anti-tag antibodies and nickel would select for binding polypeptides with associated mRNA which can then provide the gene sequence encoding for binding polypeptides. Alternatively, using ribosome display, only the polypeptide may be tagged, for example with a polyhistidine tail, passed over affinity matrices such as nickel chelate and any associated RNA may be accessed simply by converting into cDNA copies by PCR. For selection of 2 or more binding polypeptides, a particularly favourable method is to use the technique of ribosome display using di(or poly)cistronic mRNAs for generation of candidate binding polypeptides derived from one or more DNA libraries. If the cistron for one of the polypeptides is constructed such that the translated polypeptide is dissociated from mRNA after translation and the cistron for the other polypeptide is constructed such that the translated polypeptide remains associated with mRNA after translation, then the translational juxtaposition of the 2 polypeptides is such that association, if applicable, is facilitated by this juxtaposition thus providing a molecular basis for separation of the complexes including the binding polypeptide(s) and mRNA, the latter which can then be used for the identity of genes encoding the binding polypeptide(s).

Another embodiment of the present invention is based upon the binding of 2 polypeptides, one or both of which are associated with the corresponding gene, which are fused (or associated) with other polypeptide chains which, when juxtaposed, will generate a selectable property which then allows isolation of live microorganisms encoding the gene(s) for the binding polypeptides(s). Of particular interest within this embodiment is the generation of enzyme activities by the association of 2 polypeptides. Such generated enzyme activities can be used in a variety of ways for selection. These include enzyme activities which generate molecular tags which attach to complexes of successfully paired polypeptides and associated genes (or mRNA). For polypeptide display on microorganisms, these also include enzyme activities which generate (or release) molecules in the vicinity of the microorganisms which leads to the selection of microorganisms displaying a polypeptide derived from a DNA library which binds to a ligand. Also of interest within this embodiment is the generation of binding protein activities by the association of 2 polypeptides whereby the binding protein indirectly generates a selectable property which can be used as a basis for identifying the gene encoding the binding protein(s). For example, the binding protein could be a DNA binding protein such as a transcriptional activator which activates a gene which provides a selection advantage to the microorganism such as an antibiotic resistance protein or a nutrient generating protein.

For application of the present invention to binding polypeptides displayed on

For application of the present invention to binding polypeptides displayed on microorganisms, the invention provides for methods where the ligand (or second polypeptide) is added externally to the microorganism or methods where the ligand is synthesised internally within the microorganism, in both cases where the association of the polypeptide and ligand can generate a new molecule (or molecules) which provides a basis for selection or separation of the microorganism displaying the binding polypeptide. The invention can be based upon the complementation of an enzyme activity through the combination of 2 (or more) subunits of an enzyme which, when associated together, reform the enzyme activity. Reformation of the enzyme activity will then either provide for the positive or negative selection of the microorganism providing the enzyme activity. In one example of this embodiment of the invention, there is provided a DNA library encoding variant polypeptides which can be expressed in a microorganism or a cell whereby the genes encoding the binding polypeptides are fused to a portion of the gene encoding an enzymatically inactive fragment of an enzyme whereby this portion of enzyme can complement another portion of the enzyme to reconstitute enzyme activity. Suitable enzymes include *E. coli* beta-galactosidase and *C. perfringens* phospholipase C. The other portion of the enzyme is either provided in the microorganism culture medium for selection *in vitro* or provided by expression of a gene encoding this portion within the same microorganism for selection *in vivo*. Enzymes such as *E. coli* beta-galactosidase and *C. perfringens* phospholipase C have the property that inactive subunits of the enzyme can self-associate in solution to reconstitute enzyme activity and thus the invention provides for a situation whereby the binding polypeptide, fused to the gene encoding an inactive enzyme fragment, can bind to its ligand and either promote (where the ligand is fused to the other inactive subunit) or prevent (where the polypeptide-ligand binding, through steric hindrance, prevents the reconstitution of enzyme activity by the inactive enzyme subunit. The restoration or abolition of enzyme activity may have a beneficial or deleterious effect on the microorganism expressing the polypeptide / enzyme subunit gene fusion. This will either be due to the direct enzymatic action of the reconstituted enzyme, for example in causing the conversion of a non-toxic substrate to a toxic product, or indirect enzymatic action of the reconstituted enzyme, for example in causing the lysis of external liposomes containing a toxic agent or an essential nutrient/factor for growth/propagation of the microorganism.

Yet another embodiment of the invention is based upon the binding of a polypeptide with a non-protein or defined protein moiety, whereby molecular tags on the polypeptide or the non-protein or defined protein moiety can be used as the basis for isolation of interacting polypeptide with non-protein or defined protein moieties from the unbound polypeptide and non-protein or defined protein moiety whereby the polypeptide is still associated with its gene (or gene transcript). The non-protein moiety may be defined as a molecule which is composed, in the main, of non-amino acids constituents such as nucleic acids (DNA or RNA) or chemicals (synthetic or natural). The defined protein moiety will be a protein the identity of which is known, for example, a preparation of total serum immunoglobulin whereby the serum immunoglobulin might interact with certain of the polypeptides. Association of the polypeptide with its gene transcript would be achieved using the technique of ribosome display where the polypeptide is part of a nascent protein which remains attached to the ribosome/mRNA complex, where the mRNA itself may be tagged with a RNA binding protein (such as HIV tat protein) oligonucleotide conjugated (or linked

a RNA binding protein (such as HIV tat protein) oligonucleotide conjugated (or linked to) a non-protein or defined protein moiety, or where the mRNA may be tagged with a hybridising synthetic oligonucleotide conjugated (or linked to) a non-protein or defined protein moiety. Juxtaposition of the protein and non-protein or defined protein moiety can be achieved by attaching the non-protein moiety to a synthetic oligonucleotide which hybridises to the mRNA molecule. The non-protein or defined protein moiety may alternatively be tagged with, for example, a polyhistidine tail whilst the polypeptide moiety is linked to the mRNA as previously described such that consecutive passages of the library of mRNA-ribosome-polypeptide and non-protein or defined protein moiety complexes over affinity matrices comprising anti-tag antibodies and nickel would select for the bound polypeptide with it's associated mRNA. For example the non-protein moiety could be DNA and the bound polypeptide a DNA-binding protein or in another example the defined protein moiety may be a lymphokine and the bound polypeptide a lymphokine receptor or region thereof.

Attachment of the non-protein or defined protein moiety to a hybridising oligonucleotide or mRNA binding protein could be achieved by any of the conventional ways of cross-linking polypeptides, such as those generally described in O'Sullivan et al., (Anal. Biochem 100, (1979), 108). Such cross-linking may be facilitated by the introduction of specific amino acids into the defined protein or the mRNA binding protein, especially free cysteine residues or free lysine residues. Alternatively attachment of the defined protein moiety may be achieved by the incorporation of biotin into both the hybridising oligonucleotide or mRNA binding protein and the non-protein or defined protein moiety (as described by Langer et al., PNAS 78, (1981), 6633) followed by the addition of avidin to cross-link the oligonucleotide or mRNA binding protein to the non-protein or defined protein moiety.

Still another embodiment of the present invention provides for the screening for agonists and antagonists of defined biochemical pathways whereby the individual proteins comprising the components of the pathway would be sequentially expressed as full length polypeptides which remain attached to the polycistronic mRNA via a stalled ribosome. For example, using the technique of ribosome display, the proteins comprising a biological pathway could be sequentially cloned into an appropriate vector and displayed. Interaction between the polypeptides facilitated by close physical proximity will result in a detectable change in the final polypeptide in the pathway. For example the sequential association of the polypeptides will result in a phosphorylation cascade which causes a terminal phosphorylation or other event such as a conformational change. A particularly favourable method for the detection of the phosphorylation or conformation of the final polypeptide in both activated and non-activated states would be using antibodies. For selection of antagonists of the pathway, a particularly favourable method is to use the technique of ribosome display using polycistronic mRNA for the display of proteins in a biochemical pathway, sequentially juxtaposed. The displayed proteins are then screened with candidate molecules, whereby the candidate molecules may for example be ribosome displayed polypeptides derived from one or more DNA libraries, and inhibition of the activity of the pathway proteins assayed.

The following example illustrates the invention but should not be considered to limit the scope of the invention:

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Example 1

The self-dimerising human IL-5 protein was used to demonstrate complementation of phospholipase C (PLC) from it's two inactive subunits, PLC1-246 (PL1) and PLC247-370 (PL2) and subsequent lysis of liposomes. A human IL-5 gene (R&D Systems, Abingdon, UK) was PCR amplified from pUC18 using the following primers:

il5f1: CCG TAT AGA TCT GAA ATT CCC ACT AGT GCA TTG
*Bgl*II

il5r1: CCG TAT GGA TCC GAC GTC CTC AAG CTT GGA ATA TTA TCA
*Bam*HI *Hind*III Stop
-CCC GGG ACT TTC TAT TAT CCA CTC GGT
*Sma*I

il5r2: CCG TAT GGA TCC GAC GTC CTC AAG CTT GGA ATA
*Bam*HI *Hind*III
-CCC GGG CCA TGG ACT TTC TAT TAT CCA CTC GGT
*Sma*I *Nco*I

As a control for IL-5, a single chain antibody genes (scFv) specific for digoxin (Tang *et al.*, Journal of Biological Chemistry **270** (1995) p7829-7835) was also cloned starting from a pCANTAB vector (Tang *et al.*) using forward primer, fdig1: CCG TAT AGA TCT CAG GTC AAA CTG CAG GAG TCT and reverse primer, rdig1: CCG TAT GGA TCC CCG TTT TAT TTC CAA CTT TGT.

PCR amplification reactions were performed using the Boehringer Expand High Fidelity PCR system (Boehringer, Lewes, UK). Reaction conditions for amplification of DNA fragments were 1X Expand HF buffer, 2.5 mM MgCl₂, 4 mM of each dNTP, 2.5 units of polymerase, 10 ng template DNA and 30 pmol of primer DNA. Reactions were incubated in a thermal cycler using the following programme: 92°C for 5 min, 53-67°C (depending on primer sequence) for 5 min, 72°C for 1 min, followed by 30 cycles of 92°C for 1 min, 53-67°C for 1 min and 72°C for 1 min. The resultant fragments were then purified using Wizard PCR purification columns (Promega) and cloned into the *E. coli* expression vector pET-9 (Promega, Southampton, UK) at the *Bam*HI site by digesting the PCR product and vector with *Bgl*II and *Bam*HI according to the manufacturer's instructions. This plasmid contains the promoter, translational start site and terminator from the bacteriophage T7 gene 10. The resultant plasmids were designated pIL5a (*Sma*I site) and pIL5b (*Sma*I and *Nco*I sites) or pDIG (anti-digoxin scFv).

pIL5b and pDIG were further modified as follows: A spacer sequence based on the glycine rich linkers of gene III of filamentous phage M13 was generated by performing PCR on a preparation of double stranded M13 DNA using the following two sets of primers:

m13f1: CCG TAT AGA TCT GGCTTTAATGAGGATCCATTC
*Bgl*III

m13r1: CCG TAT CTC GAG CTGTAGCGCGTTTTTCATCGGC
*Xho*I

m13f2: CCG TAT GTC GAC GGCTTTAATGAGGATCCATTC
*Sal*I

m13r2: CCG TAT TGA TCA CTGTAGCGCGTTTTTCATCGGC
*Bcl*II

Two sets of PCR reactions were performed using primer combination m13f1 and m13r1 or with m13f2 and m13r2. These two sets of reactions generated two populations of products, one with a 5' *Bgl*III and 3' *Xho*I and one with a 5' *Sal*I and 3' *Bcl*II restriction sites. The restriction sites were included to facilitate the construction of multimers of the 30 amino acid linker. The *Bgl*III / *Xho*I PCR products were double digested and phosphatased and then ligated with the digested *Sal*I/*Bcl*II PCR products. In this way multimers ligated only 5' to 3' (which could be confirmed by digestion) would be formed. A 900 bp fragment was isolated by agarose gel electrophoresis and purified using Wizard PCR purification columns (Promega) according to manufacturers instructions. The HIV transactivation response element (TAR) sequence was inserted downstream from the M13 spacer segment by ligating the *Bcl*II digested 900 bp spacer fragment (generated as described above) to a self annealed oligonucleotides encoding the HIV TAR as follows:

TAR1: GATCAGCCAGATTTGAGCAGC
TAR2: GATCGCTGCTCAAATCTGGCT

This fragment was repurified, digested with *Bgl*III and cloned into the *Bam*HI site of pIL5b or pDIG too give pIL5b/M13/TAR and pDIG/M13/TAR respectively.

The PLC-encoding plasmid pT2.2 (Titball R et al., Infection and Immunity, 57 (1989) p367-376) was subjected to PCR (conditions as above) using the following primer pairs;

PL1for: CCG TAT CCA TGG GGA TGG AAA GAT TGA TGG AAC

PL1bck: CCG TAT CCC GGG GAT ACA TCG TGT AAG AAT CTA

PL2for1: CCG TAT CCC GGG TAA TGA TCC ATC AGT TGG AAA

PL2for2: CCG TAT AGA TCT TAA TGA TCC ATC AGT TGG AAA

PL2bck1: CCG TAT AAG CTT TTA TTT TGT AAA TAC CAC C

PL2bck2: CCG TAT GAA TTC AAG CTT TTA TTT TGT AAA TAC CAC C

The PL1 fragment from PCR with PL1 primers was digested with *NcoI* and *SmaI* and cloned into *NcoI/SmaI*-digested pIL5b(or pDIG)/M13/TAR to give pIL5(pDIG)/PL1/M13/TAR. The PL2 fragment from PCR with PL2for1 and PL2bck1 primers was digested with *SmaI* and *HindIII* and cloned into *SmaI/HindIII*-digested pIL5a to give pIL5/PL2. The PL2 fragment from PCR with PL2for2 and PL2bck2 primers was digested with *BglII* and *BamHI* and cloned into *BamHI* digested pET-9 to give pPL2.

In vitro transcription of the plasmid constructs was performed using the RiboMAX large scale RNA production system(Promega) according to the manufacturers instructions. The resultant mRNA was purified using PolyATtract system (Promega).

For the liposomal-tat preparation, liposomal vesicles were produced by mixing the lipids L-phosphatidylcholine and oleoyl-palmitoyl cholesterol. Tat 37-72 peptide at a concentration of 100mM in 10mM Tris pH8.0 was added to 10mg of a mixture of 1.5:1 L-alpha-phosphatidylcholine and cholesterol (Sigma Poole U.K; #13906). Vesicles were formed following repeated cycles of vigorous mixing and allowing the solution to stand at room temperature. The volume of the liposomal solution was increased by addition of 10mM Tris 10mM pH8.0 and liposomes purified from unincorporated components by gel filtration through a G25 Sephadex column (Pharmacia, Milton Keynes, UK; #17-0851-01) with borate buffered saline (BBS; 0.2M sodium metaborate, 7.5g/l NaCl, 1.8g/l CaCl₂·2H₂O, pH adjusted to 7.0 with boric acid). Integrity of the eluted liposomes was assessed by microscopy where intact liposomes were compared to control preparations lysed by treatment with a solution of 1% (v/v) NP-40 (Pierce, Chester, U.K.; #28324). Liposomes were diluted in BBS to give a final concentration of 10mM tat 37-72 peptide.

Anti-tat antibody was made using the tat peptide 37-72 which was conjugated via its N-terminal cysteine residue to KLH using MBS according to *Antibodies, A Laboratory Manual*, eds Harlow E. & Lane D., Cold Spring Harbor. 10ug of the conjugate was used to immunise Balb/c mice as above and serum was collected and used at 1:100 dilution in the further experiments.

In vitro translation was performed in an *E. coli* S-30 system as described by Chen and Zubay (ibid) modified as described by Hanes and Pluckthun (ibid). The translation was stopped and the mixture centrifuged as described by Hanes and Pluckthun (ibid).

100ul of prepared liposomal-tat suspension was added to 100ul translation mixture and incubated for 1 hour at 37°C. Translation reactions were then passed through a column comprising polyclonal anti-tat antibody on protein A beads prepared according to *Antibodies, A Laboratory Manual* ibid. Washing and dissociation of retained ribosome complexes, isolation of mRNA, reverse-transcription PCR and repeated transcription-translation were as described by Hanes and Pluckthun (ibid).

Aliquots of mRNA derived from either of the plasmids pIL5a, pIL5a/PL2 or pPL2 (encoding the "model ligands" IL-5, IL-5/PL2 fusion protein or PL2 alone) and rabbit beta globin mRNA ("globin") were mixed in a 1:1 w/w ratio with a 1:9 w/w mixture of mRNAs derived from the plasmids pIL5b/PL1/M13/TAR and pDIG/PL1/M13/TAR (encoding the "model receptors" IL-5/PL1 fusion protein or anti-digoxin scFv/PL1 fusion) for subsequent translation and separation of tat peptide-tagged mRNAs. All reactions were in triplicate. The identity of the mRNAs (IL-5 or DIG) were subsequently determined from a minimum of 50 clones in pUC18. The results of this analysis are shown in table 1 (average of 3 determinations; SD < 10%) and indicate that the use of IL-5/PLC ligand provides a strong selection of mRNAs encoding IL-5 in preference to anti-digoxin scFv. This indicates that the homodimerisation of IL-5 brings together the PL1 and PL2 subunits of PLC for efficient lysis of liposomes and release of tat which binds to anti-IL5 mRNA in the translation complexes associated with IL-5 dimers.

Model Ligand	% IL5 clones
Globin	8
IL-5	12
IL-5/PL2	100
PL2	18

Table 1:

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